Posttraumatic therapeutic hypothermia alters microglial and macrophage polarization toward a beneficial phenotype

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Abstract
Posttraumatic inflammatory processes contribute to pathological and reparative processes observed after traumatic brain injury (TBI). Recent findings have emphasized that these divergent effects result from subsets of proinflammatory (M1) or anti-inflammatory (M2) microglia and macrophages. Therapeutic hypothermia has been tested in preclinical and clinical models of TBI to limit secondary injury mechanisms including proinflammatory processes. This study evaluated the effects of posttraumatic hypothermia (PTH) on phenotype patterns of microglia/macrophages. Sprague-Dawley rats underwent moderate fluid percussion brain injury with normothermia (37°C) or hypothermia (33°C). Cortical and hippocampal regions were analyzed using flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR) at several periods after injury. Compared to normothermia, PTH attenuated infiltrating cortical macrophages positive for $CD11b^+$ and $CD45^{high}$. At 24 h, the ratio of $iNOS^+$ (M1) to arginase$^+$ (M2) cells after hypothermia showed a decrease compared to normothermia. RT-PCR of M1-associated genes including iNOS and IL-1β was significantly reduced with hypothermia while M2-associated genes including arginase and CD163 were significantly increased compared to normothermic conditions. The injury-induced increased expression of the chemokine Ccl2 was also reduced with PTH. These studies provide a link between temperature-sensitive alterations in macrophage/microglia activation and polarization toward a M2 phenotype that could be permissive for cell survival and repair.

Introduction

Traumatic brain injury (TBI) is a major worldwide clinical problem.[1] Although a significant amount of information is known regarding the pathophysiology of brain injury, the successful translation of therapeutic interventions to the clinic is yet to be achieved.[2] One therapeutic intervention that has shown promise in preclinical investigations as well as in several patient populations including cardiac arrest, cerebral ischemia, neonatal hypoxic encephalopathy, and brain and spinal cord trauma is therapeutic hypothermia and targeted temperature management.[3,4] In the area of experimental TBI, multiple laboratories using various injury models and outcome measures have reported the benefits of early cooling on histopathological and behavioral outcomes.[5] Therapeutic hypothermia significantly reduces histopathological damage caused by brain injury by targeting multiple secondary injury mechanisms including excitotoxicity, apoptosis, inflammation, and elevations in intracranial pressure.[5,6]

Posttraumatic inflammation is an important secondary injury mechanism after TBI and a target for therapeutic interventions.[7–9] Recently, a role for inflammatory mechanisms in the generation of chronic

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neurodegenerative processes associated with TBI has also been emphasized.[10–12] The activation profiles of macrophages and microglia after experimental and clinical TBI are complex and include early and prolonged alterations in experimental and clinical studies.[10,12,13] Posttraumatic inflammatory processes can promote secondary neuronal cell death and circuit dysfunction by the generation and release of proinflammatory and cytotoxic mediators including reactive oxygen species and proinflammatory cytokines and chemokines.[9,14] In this regard, preclinical studies have tested anti-inflammatory treatments or used transgenic models to target inflammatory mediators to promote neuroprotection and improve functional outcomes.[7] Unfortunately, to date no pharmacologic agents have demonstrated efficacy in Phase III clinical trials for TBI.[2]

The beneficial effects of posttraumatic hypothermia (PTH) are associated with the attenuation of a variety of secondary inflammatory processes including the reduced expression and levels of inflammatory proteins, metalloproteinases, and other potentially cytotoxic agents.[5,15] PTH reduces blood–brain barrier (BBB) breakdown, the activation of resident microglia, as well as attenuating the infiltration of circulating blood monocytes into the brain parenchyma.[16,17] Although single institutional therapeutic hypothermia studies for severe TBI showed beneficial effects on reducing elevated intracranial pressure and improving clinical outcomes, the failure of recent multicenter trials has reemphasized the importance of patient selection and cooling protocols.[3]

In addition to the detrimental consequences of secondary inflammatory processes, host immunological responses can also be beneficial in clearing necrotic debris, promoting wound repair, and participating in the synthesis and release of growth-promoting substances.[18,19] For example, it is known that microglia can influence the proliferation, differentiation, and survival of neuronal precursor cells as well as promote angiogenesis.[20,21] To help explain the apparently divergent roles of inflammation in central nervous system injury, emerging evidence indicates that inflammatory cells are relatively plastic after injury leading to subsets of microglial and macrophage phenotypes that may have distinctive beneficial and detrimental effects on outcomes.[13,19,22] Although somewhat simplistic in nature, studies have described different states of microglia/macrophage polarization that help clarify these divergent processes.[23,24] Following brain or spinal cord trauma for example, an M1 macrophage response which indicates "classical activation" and felt to be cytotoxic is rapidly induced and maintained at sites of injury.[18,19,22] In contrast, the M2 macrophage

"alternative response" which is thought to be more reparative is less pronounced and only transiently expressed after brain injury.[11,19,25] After controlled cortical impact injury, Ansari[25] recently reported that the M1 response was significantly elevated in the hippocampus at 2 h and was significantly larger than the comparatively smaller transient M2 response.

With postinjury phenotype plasticity in mind, research including pharmacological or adoptive transfer strategies is being advanced to promote a shift in inflammatory profiles from a M1-dominated to M2-like phenotype after CNS injury.[18,19,26,27] These investigations are helping to clarify specific strategies to help balance the different inflammatory cell phenotypes to maximize protection and repair after CNS injury.[19] Although the effects of PTH on the early inflammatory response to TBI have been demonstrated, no studies have determined whether therapeutic hypothermia alters the balance of M1-/M2-like polarization of microglia and macrophages toward a pro-regenerative phenotype.

For this question we utilized a moderate fluid percussion injury model and a therapeutic hypothermia paradigm that has previously been shown to improve histopathological and behavioral outcomes.[3] We evaluated the temperature sensitivity of functionally distinct subsets of microglia and macrophage phenotypes using cell sorting, flow cytometry, as well as quantitative RT-PCR. Together our findings indicate that PTH not only attenuates overall microglia/macrophage activation but also produces significant effects on phenotype polarization.

Methods

Animals

Male Sprague-Dawley rats weighing between 280 and 340 g obtained from Charles River Breeders were used for all experiments. Animal care was in accordance with the guidelines set by the University of Miami Animal Care and Use Committee and the NIH *Guide for the Care and Use of Laboratory Animals*, in compliance with the Animal Research Reporting in In Vivo Experiments guidelines. All animal protocols were approved by the University of Miami Animal Care and Use Committee prior to study initiation. Reporting of the results conforms to the Animal Research Reporting in In Vivo Experiments guidelines. All animals were kept at a constant temperature (23–25°C) in an air-conditioned room for at least seven days before the study and exposed to a 12 h light–dark cycle. Rats were allowed free access to water, but food was withheld overnight before injury.

Surgical preparation and fluid percussion injury

Anesthesia was induced using 1–3% isoflurane in a gas mixture of 70% nitrous oxide and a balance of oxygen, to achieve deep sedation. Animals were placed in a stereotactic frame for introduction of a right parietal cortex craniotomy (4.8 mm in diameter) positioned 3.8 mm posterior to bregma and 2.5 mm lateral to the midline leaving the dura intact. The plastic injury tube (2.6 mm diameter) was positioned over the exposed dura and bonded by adhesive. The scalp was sutured closed and the animals were returned to their home cages and allowed to recover.

After fasting overnight, a fluid percussion device was used to produce experimental TBI through the injury tube.[17] The animals were again anesthetized, endotracheally intubated, and mechanically ventilated with a Harvard rodent ventilator adjusted as described below. Femoral artery and vein were cannulated with PE-50 tubing for purposes of drug administration, blood sampling for serum glucose and hematocrit measurements, arterial blood gas determination, and continuous arterial blood pressure monitoring. Anesthetic concentrations and ventilatory settings were adjusted to maintain normal values of mean arterial pressure, pH, pO_2, and pCO_2. Rectal and temporalis muscle thermometers were placed, and self-adjusting feedback external warming lamps were used to maintain temperature. Arterial blood gases, blood glucose, and hematocrit were measured 15 min before and after fluid percussion brain injury, and 1, 2, 3, and 4 h after TBI. Pancuronium bromide 0.5 mg/kg i.v. was administered every hour during the surgical procedure to facilitate mechanical ventilation. In TBI animals, a moderate level of injury (1.8–2.2 atm) was produced, as previously described.[17] Sham-operated animals underwent all surgical procedures but were not injured.

Temperature manipulation

Rectal and temporalis muscle thermistors measured core and brain temperatures, respectively, using self-adjusting feedback warming lamps. The post-TBI brain temperature was maintained for 4 h at normothermia (37°C) or mild (33°C) hypothermia. Posttraumatic brain hypothermia was achieved within 30 min following TBI by blowing cooled air directly onto the skull with a small fan. At the end of the cooling period, the animals were either sacrificed by decapitation (4 h group) or slowly rewarmed to normothermic temperature and allowed to survive. Animals were randomized to either normothermic or hypothermic groups and group size determined based on previous TBI experiments using similar techniques.

All outcome assessments were conducted by an investigator blinded to the experimental groups.

Flow cytometry

Flow cytometry was performed to differentiate resting microglia from activated microglia and macrophages. At 4 h, 24 h, 3d, or 7d after TBI (n = 6 per group), animals were perfused with cold PBS and ipsilateral parietal cortex and hippocampus dissected for analysis. Single cell suspensions were made by passage through a 70 μm cell strainer (Falcon, Madison, WI), lysed with ACK buffer (Life Technologies, Grand Island, NY), and stained with Live/Dead reagent (Life Technologies). Following a nonspecific block with CD16/CD32 antibody, cells were incubated with fluorescent antibodies for surface markers CD11b (v450, BD Horizon, San Jose, CA) and CD45 (Alexa 647, Biolegend, San Diego, CA) to separate microglia (CD11b+, CD45^{low}) and infiltrating leukocyte (CD11b+, CD45^{high}) populations. Cells were also incubated with RP1 (PE, BD Horizons) to identify granulocytes. After fixing, cells were incubated with antibodies for iNOS (FITC, eBioscience, San Diego, CA) and Arginase (PE, R&D Systems, Minneapolis, MN) antibodies to separate those activated cells that display either the M1 (iNOS+) or M2 (Arg+) phenotype as analyzed on a flow cytometer (BD FACSDiva LSRII) using Kaluza software for analysis. All samples were also incubated with isotype controls for each antibody. Experimental groups analyzed for iNOS and Arginase included TBI normothermia (37°C) and TBI hypothermia (4 h 33°C) with survival times of either 4 or 24 h.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed on ipsilateral cortex and hippocampus from TBI normothermia and TBI hypothermia (4 h) animals. Sham operated or contralateral cortex or hippocampus from normothermic animals was used as the control. Four hour survival (n = 5) and 24 h survival (n = 5) animal groups were assessed for several genes associated with M1 or M2 microglia/macrophage phenotypes. Tissue was fresh frozen after decapitation and RNA was extracted using Direct-zol^{TM} (Zymo Research, Irvine, CA) kit. After quantification of RNA concentration, equal amounts (1 μg) of RNA were reverse transcribed using the RT2 First Strand Kit (SABioscience, Frederick, MD) which includes a genomic DNA removal step. Real-time qPCR was done using Sybr green dye and gene-specific primers (SA Biosciences) performed on the Applied Biosystems 7300 Real Time platform (Foster City,

CA). Primers for markers of M1 phenotype included IL-1β, IL-1α, IL-6, IL-12, TNFα, and iNOS. M2 phenotype-associated genes included Arginase, CD206, CD163, YM1 (Chil3), TGFβ, and IL-10. RT-PCR was done for the chemokine Ccl2 (formally known as monocyte chemoattractant protein 1, MCP1) as well. Ccl2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize samples. Each sample was run in triplicate and relative expression levels were analyzed using the 2^{-ddCt} method.

Statistics

Flow cytometry and RT-PCR data were analyzed using one-way analysis of variance followed by Holm Sidak or Student–Newman–Keuls post hoc comparison. Data are expressed as mean ± SEM.

Results

Animal physiology

Physiological measurements of rectal and brain temperature. PCO_2, PO_2, pH, and mean arterial blood pressure are summarized in Table 1. Physiological variables were taken at 15 min prior to TBI and 15 min, 1, 2, 3, and 4 h after injury. Physiological variables were within normal ranges. The only significant difference between groups was seen in the temporalis muscle temperature where the hypothermic group had significantly reduced temporalis temperature compared to that of the normothermic TBI animals as predicted (*4 h $p < 0.001$; **24 h $p < 0.01$).
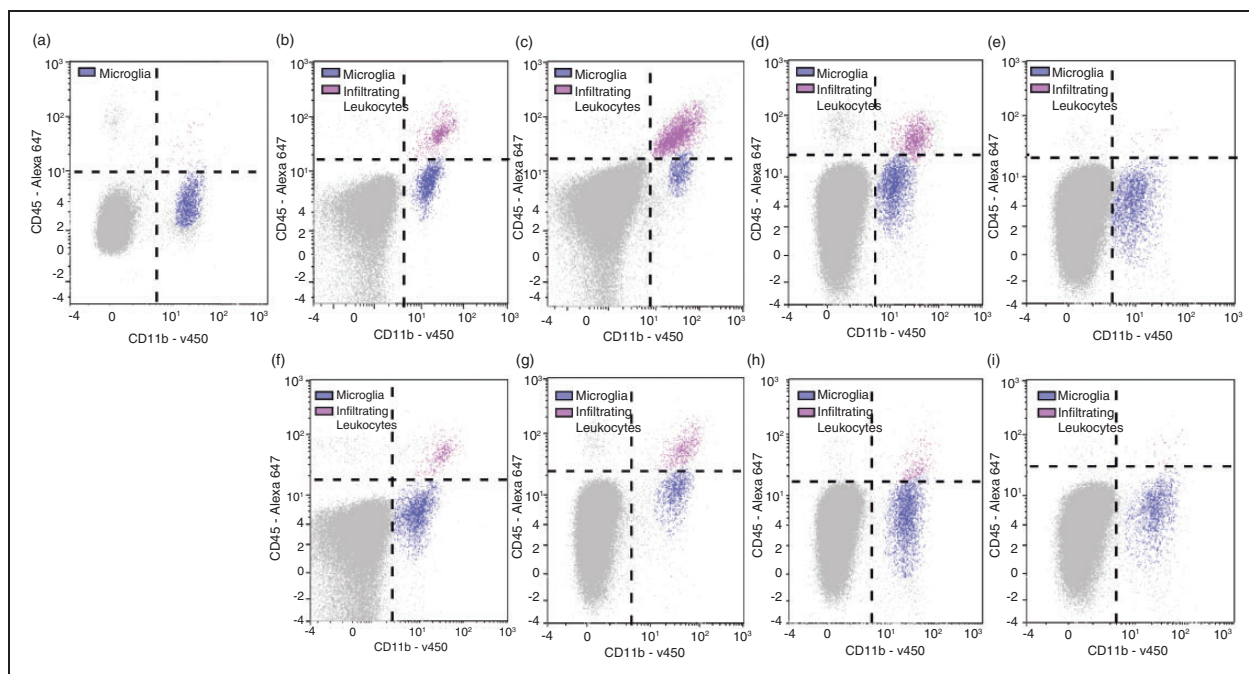
Flow cytometry

Flow cytometry was used to separate populations of resident microglia and infiltrating cells including neutrophils and macrophages. Cells were labeled with CD11b which is a cell surface marker for neutrophils, macrophages, and microglia. Additionally, cortical cells were labeled with CD45 which is a cell surface marker for leukocytes. The cells that were positive for CD11b but not for CD45 included resident microglia and those that were positive for both CD11b and CD45 indicated infiltrating neutrophils and monocytes, including macrophages (Figure 1(a) to (i)). A time course of 4 h, 24 h, 3d, and 7d showed that there were no infiltrating cells in the sham-operated animals (Figure 1(a)). Starting at 4 h and increasing to 24 h, a significant increase in infiltrating neutrophils and macrophages was observed in the cortex (Figure 1, upper right quadrant of flow cytometry-gated populations). The number of CD45 high expressing cells began to decrease at three days and was reduced to sham levels by seven days in cortex (Figure 2(a) to (d)). This is consistent with a transient disruption of the BBB. In addition, 4 h of PTH attenuated the amount of infiltrating cells at 4 h (Figure 2(a), $p < 0.005$). Hypothermia also attenuated the number of infiltrating cells in the hippocampus at 4 h (Figure 3(a), $p < 0.01$) but not 24 h (Figure 3(b)).

CD11b-positive cells were also labeled with intracellular markers for M1 (iNOS) and M2 (arginase) phenotypes. Cells that were positive for CD11b but negative

Table 1. Physiological variables.

	4 h normothermia	4 h hypothermia	24 h normothermia	24 h hypothermia
	15 min pre-TBI	*15 min pre-TBI*	*15 min pre-TBI*	*15 min pre-TBI*
Weight	341.6 ± 19.58	363 ± 22.33	333.2 ± 17.66	351.2 ± 11.67
Brain temperature	37 ± 0	37 ± 0	36.78 ± 0.05	36.78 ± 0.02
pH	7.41 ± 0.01	7.40 ± 0.02	7.42 ± 0.02	7.43 ± 0.01
pCO_2	39.27 ± 2.03	41 ± 1.59	41.4 ± 1.96	40.74 ± 0.86
pO_2	167.8 ± 7.75	174.4 ± 11.62	176.6 ± 13.74	180.2 ± 9.08
MAP	133.37 ± 6.80	140.57 ± 7.02	117.96 ± 7.76	121.76 ± 7.09
	4h post-TBI	*4h post-TBI*	*15 min post-TBI*	*15 min post-TBI*
Brain temperature	37 ± 0	**33.62 ± 0.58***	36.78 ± 0.05	**33.48 ± 0.02****
pH	7.42 ± 0.02	7.41 ± 0.01	7.43 ± 0.02	7.43 ± 0.01
pCO_2	38.1 ± 1.59	39.7 ± 1.50	39.98 ± 1.86	41.12 ± 0.69
pO_2	146.2 ± 8.44	150.8 ± 10.53	159.4 ± 15.20	149.8 ± 9.44
MAP	119.6 ± 3.95	92.4 ± 3.79	104.4 ± 4.35	100.54 ± 2.16

MAP: mean arterial blood pressure; TBI: traumatic brain injury.

Figure 1. Flow cytometry time course of microglia (CD11b+, CD45^{low} : blue) and infiltrating (CD11b+, CD45^{high} : pink) cell populations in the ipsilateral cortex after TBI. Representative samples from naïve (a), normothermia (b–e), and hypothermia (f–i) at 4 h, 24 h, 3 d, and 7 d. There are no infiltrating cells (upper right quadrants, pink) in the naïve animals (a) with an increase at 4 and 24 h (b and c). By 3 d (d) infiltrating cells are decreasing and return to naïve levels by 7 d (e). Hypothermia decreases the numbers of infiltrating cells (f–i). Gray cells are non CD11b+ live cells. TBI: traumatic brain injury. (a) naïve cortex, (b) 4 h normo cortex, (c) 24 h normo cortex, (d) 3 d normo cortex, (e) 7 d normo cortex, (f) hypo cortex, (g) 24 h hypo cortex, (h) 3 d hypo cortex, and (i) 7 d hypo cortex.

for CD45 which include microglia were separated by flow cytometry (Figure 4(a)) as were cells positive for both CD11b and CD45 which include leukocytes (Figure 4(b)). The ratio of iNOS+ to arginase+ cells showed a relative decrease in the M1 phenotype in relation to M2 phenotype in samples subjected to hypothermia among the microglia populations at 24 h after TBI (Figure 4(a), p = 0.056) with a significant decrease (p < 0.05) in the infiltrating leukocytes (Figure 4(b)). Hypothermia decreased the number of M1-type cells relative to M2 at both 4 and 24 h in leukocytes as well.

Cortical cells were labeled with RP1 which labels granulocytes and analyzed by flow cytometry at 4 and 24 h after TBI (Figure 4(c)). At 24 h after TBI, there was a significant (p < 0.05) decrease of granulocytes in hypothermia-treated animals compared to normothermia confirming a reduction of infiltrating CD11b+ cells.

RT-PCR

Ipsilateral cortical tissue was assessed for expression of multiple genes associated with either the M1 phenotype of microglia (Figure 5(a) to (f)) or the M2 phenotype

(Figure 6(a) to (f)) at 4 and 24 h after injury. Ipsilateral hippocampus was also assessed at 4 h (Supplemental Figure 1). Genes associated with the M1 phenotype included iNOS, IL1β, IL1α, TNFα, IL-6, and IL-12. The levels of expression in cortex were increased at 4 h post-TBI with a decrease in all by 24 h. iNOS and IL1β were significantly decreased at 4 h by hypothermia (Figure 5(a) and (b)), p < 0.01. Relative levels of expression for genes associated with the M2 phenotype included arginase, CD163, CD206, IL-10, YM1, and TGFβ (Figure 6). Hypothermia increased the level of expression in cortex at 4 h of all of these genes with arginase and CD163 (Figure 6(a) and (b)) reaching significance (p < 0.05) while the others showed a trend toward increasing. The hypothermia-induced effect was lost by 24 h. Levels of proinflammatory genes IL1α, IL1β, IL-12, and IL6 were increased in hippocampus relative to control after TBI at 4 h but were not altered by hypothermia (Supplemental Figure 1). M2 markers in the hippocampus at 4 h were not affected by TBI (Supplemental Figure 1).

Ccl2 expression was highly induced in the ipsilateral cortex by TBI at both 4 and 24 h relative to control
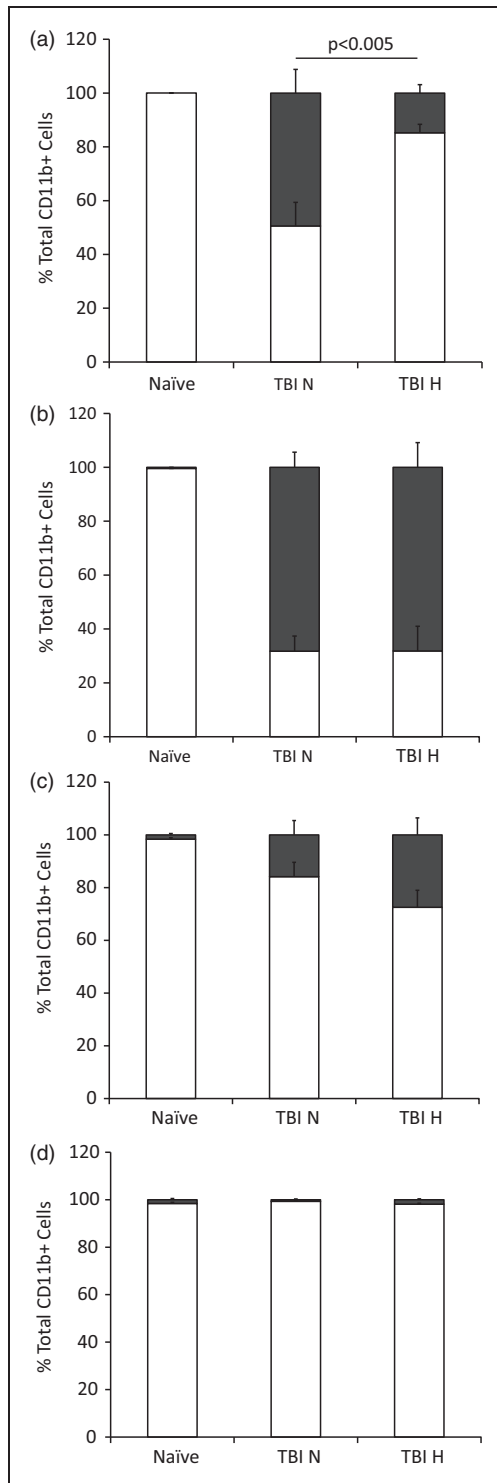
Figure 2. Flow cytometry of injured cortex at 4 h (a), 24 h (b), 3 d (c), or 7 d (d) postinjury, n = 6 per group. Percent total CD11b+ cells with high CD45 (gray) and low CD45 (white) levels. TBI N: normothermia, TBI H: hypothermia. At 4 h post injury (a), hypothermia significantly reduces the number of infiltrating cells (p < 0.005). TBI: traumatic brain injury.
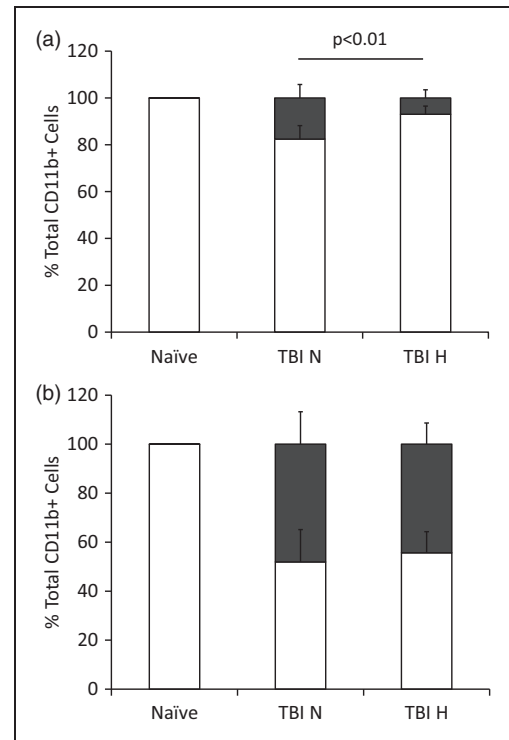

Figure 3. Flow cytometry of injured hippocampus at 4 h (a) or 24 h (b) postinjury, n = 6 per group. Percent total CD11b+ cells with high CD45 (gray) and low CD45 (white) levels. TBI N: normothermia, TBI H: hypothermia. Hypothermia significantly reduces the number of infiltrating cells at 4 h postinjury (p < 0.01). TBI: traumatic brain injury.

animals (Figure 7(a) and (b), p < 0.01). Four hours after TBI, hypothermia significantly decreased this upregulation of Ccl2 by about half (p < 0.05) in cortex. In the ipsilateral hippocampus (Figure 7(c)) at 4 h, TBI did not induce a significant upregulation of Ccl2 relative to control but interestingly, hypothermia reduced the endogenous expression of Ccl2 by about one half of the control group (p < 0.05).

Discussion

In the present study, we show for the first time that the beneficial effects of PTH are associated with the promotion of an M2 microglial/macrophage phenotype that has been proposed to be beneficial in reducing cytotoxic secondary injury mechanisms and to enhance reparative processes. We report using flow cytometry that compared to posttraumatic normothermic conditions, an induced period of hypothermia decreased intracellular makers for M1, reduced CD45 expressing leukocyte infiltration, and increased M2 markers. Quantitative RT-PCR findings supported the concept
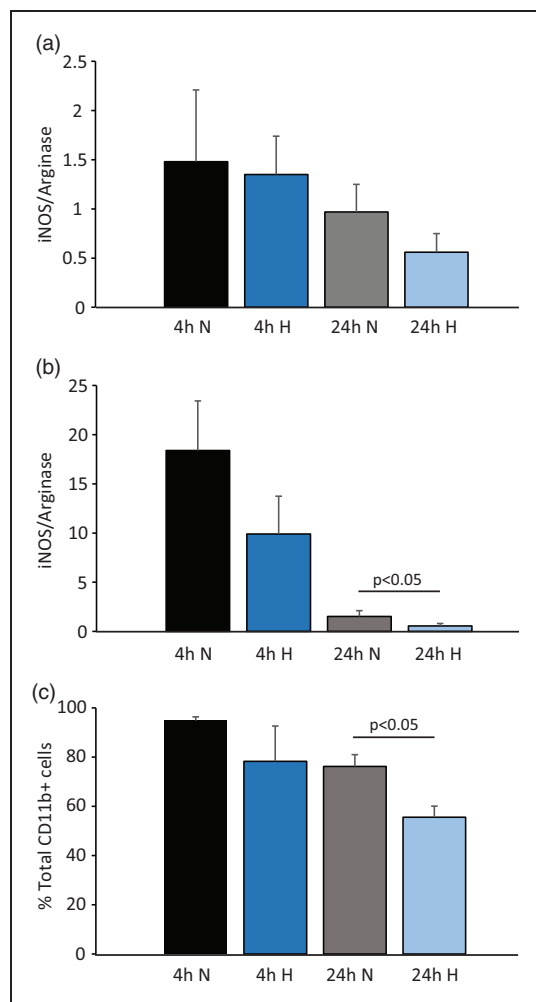
Figure 4. Flow cytometry of isolated cortical cells at 4 and 24 h after TBI, n = 6 per group. CD11b+ CD45^{low} microglia (a), CD11b+ CD45^{high} (b), and CD11b+ cells (c). Four hours of hypothermia following TBI decreases the amount of M1 microglia (iNOS+) in relation to the number of M2 microglia (arginase+) in both CD45^{low} (a, p = 0.056) and CD45^{high} (b, p < 0.05) populations. Granulocyte marker RP1 shows hypothermia decreases the % of CD11b+ cell that are positive for RP1 at 24 h (c), p < 0.05. TBI: traumatic brain injury.

of PTH enhancing an M2 phenotype by demonstrating that early cooling reduced M1-associated gene expression while increasing M2-associated gene expression. Also, the key monocyte-recruiting chemokine Ccl2 was significantly reduced by PTH. Taken together, these results demonstrate that posttraumatic temperature modifications including therapeutic hypothermia can significantly alter the M1/M2 phenotype balance, thereby having a significant effect on the inflammatory state of the posttraumatic lesion microenvironment.

Our current findings are consistent with published data showing that PTH has a significant effect on proinflammatory cytokine expression after TBI.

Truettner et al.[28] first reported using inflammatory gene arrays and RT-PCR to show that the induction of therapeutic hypothermia after TBI produced a significant but selective effect on the expression patterns of several inflammatory genes. More recently, Liu et al.[29] have reported that hypothermia after TBI decreases several proinflammatory cytokines that are associated with necroptosis including receptor-interacting protein 1 as well as mixed lineage kinase domain-like protein. Although these studies demonstrate that therapeutic hypothermia can significantly attenuate early proinflammatory genes and proteins, more limited data are available identifying cell-specific effects of therapeutic hypothermia and most likely the importance of M1/M2 polarization on long-term outcomes. In the present study, the major changes in cytokine expression were observed at the earlier posttraumatic period and not later. This may be due to the injury severity produced by the present model that produces limited cortical and hippocampal pathology thereby resulting in only transient alterations in gene expression.

Therapeutic hypothermia in the present study significantly reduced the infiltration of CD45-positive cells using flow cytometry at 4 h. CD45 is a surface marker for leukocytes and can be used to differentiate infiltrating cells from resident microglia. In addition, RP1-labeled cortical cells which identify polymorphonuclear granulocytes (PMNL) also showed a reduction in the frequency of this inflammatory population with hypothermic treatment. A reduction in the accumulation of PMNL after TBI has previously been reported with therapeutic hypothermia.[30] In another study, PTH was shown to reduce the infiltration of perivascular CD68-immunoreactive inflammatory cells at three and seven days.[17] The reduced infiltration of circulating monocytes into the injured tissue with therapeutic hypothermia may result from several mechanisms including the improved structural and functional integrity of the BBB as well as promoting a less hostile neurochemical environment within the injured microenvironment.[17,31]

The BBB plays an important role in the normal function of the CNS and has been shown to be damaged in models of brain trauma.[31,32] After focal brain injury especially, studies have documented early patterns of altered vascular permeability using a variety of protein tracers that may in some cases persist for long periods after injury.[33,34] In terms of therapeutic hypothermia, early cooling has been shown to protect BBB function in TBI models.[16,17] The extent and duration of vascular permeability results from multiple mechanisms including mechanical damage to endothelial or perivascular cells and cytotoxic factors released from damaged tissues and cells recruited to injured sites. For example, matrix metalloproteinases (MMPs) are known to
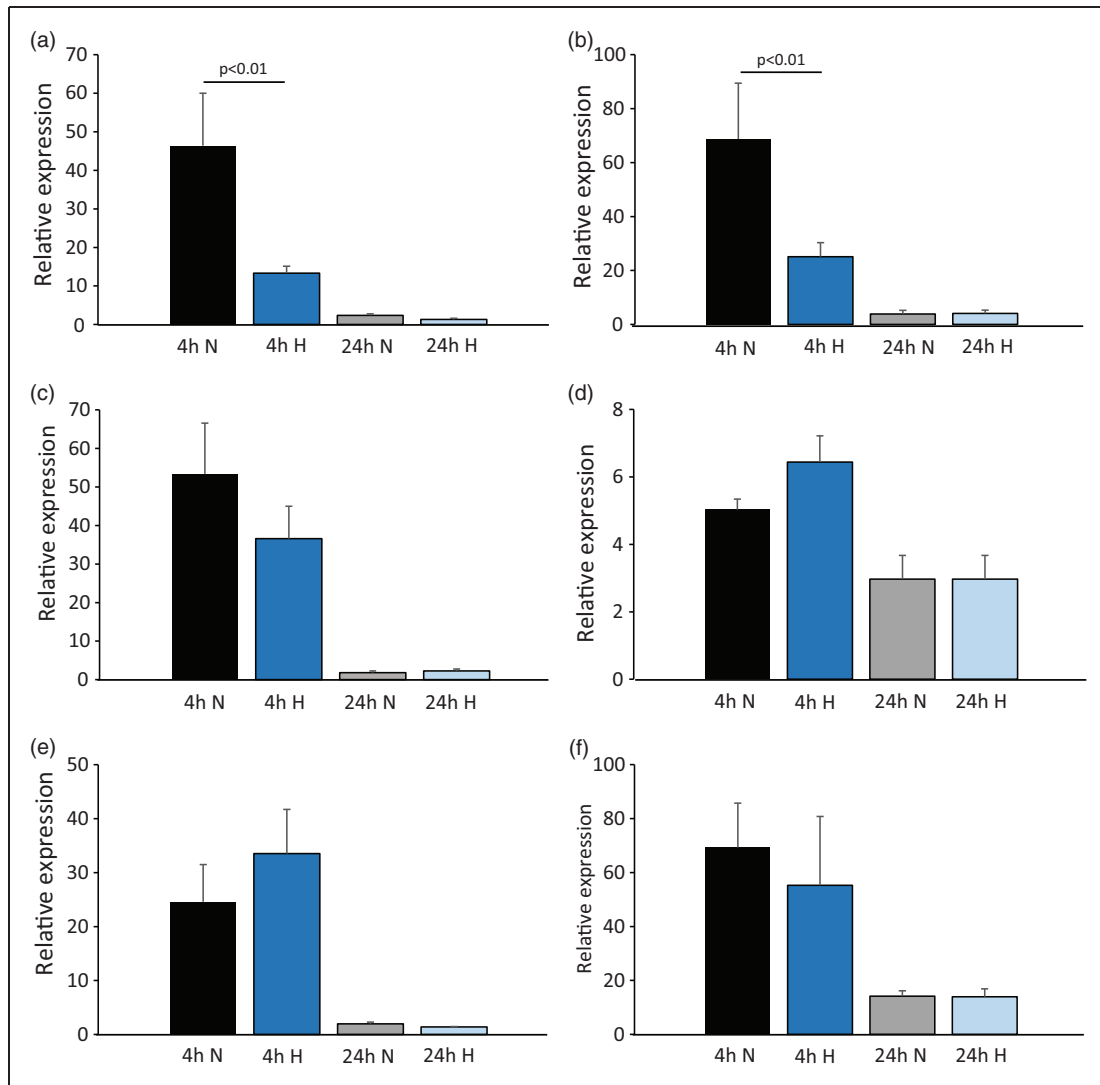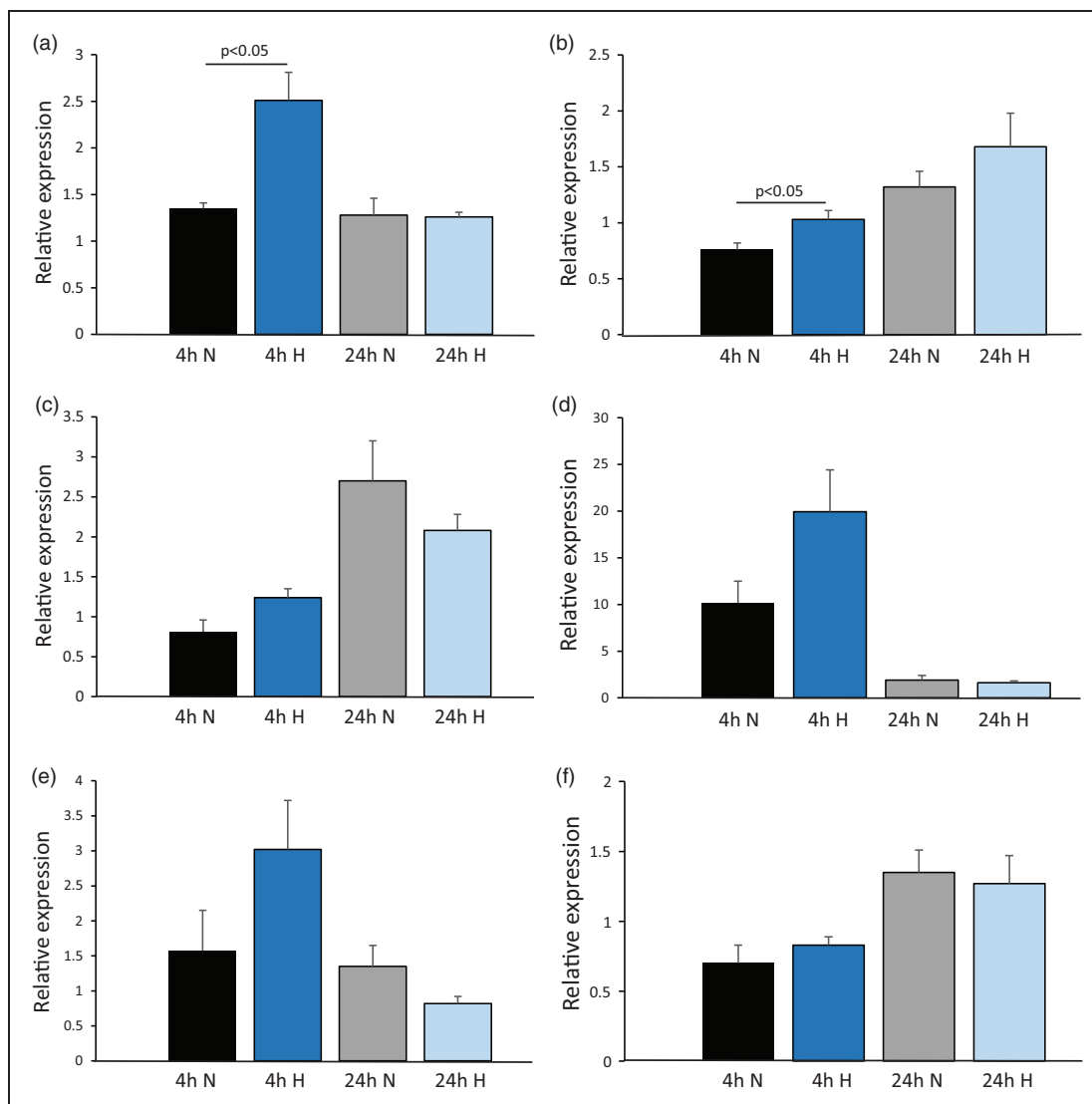
Figure 5. Relative expression of M1-associated genes by RT-PCR. Expression levels normalized to control (contralateral cortex), n = 5 per group. Expression of M1 markers iNOS (a, p < 0.01) and IL1β (b, p < 0.01) is downregulated by posttraumatic hypothermia (4 h). IL1-α (c) shows a trend at 4 h to be decreased by hypothermia although it did not reach significance and TNF-α, IL6, and IL12 (d–f) were not affected by hypothermic treatment in this experiment. RT-PCR: reverse transcription-polymerase chain reaction.

contribute to acute edema and increased vascular permeability after several types of brain injury.[31] Interestingly, increased levels of MMP-9 seen after TBI are reduced with PTH.[35]

In our study, PTH also reduced the expression of the chemokine CC ligand-2 (Ccl2) (formerly MCP-1) which has been implicated in macrophage recruitment after TBI.[36] Previous studies have reported that the genetic deletion of Ccl2 or pharmacological treatments results in the reduced macrophage recruitment deficiencies and improved outcomes in models of neurotrauma.[36,37] In a study by Liu et al.,[37] treatment with a selective antagonist to the G-protein-coupled chemokine C–C motif receptor 2 (Ccr2), the receptor that Ccl2 primarily

binds, reduced apoptotic cell death and improved behavioral outcome after TBI. PTH may therefore reduce the infiltration of CD45-positive cells by protecting the BBB and reducing the synthesis and release of cytotoxic mediators involved with the recruitment and extravasation of circulating monocytes into areas of damaged tissue.

An important finding of the present study was that PTH altered the balance of the M1/M2 phenotype compared to normothermic conditions within CD11b-positive microglia. Specifically, the ratio of iNOS-positive to arginase-positive cells showed a relative decrease in the M1 phenotype in relation to M2 phenotype with hypothermia. Microglia are an important component

Figure 6. Relative expression of M2-associated genes by RT-PCR. Expression levels normalized to control (contralateral cortex), n = 5 per group. Expression of M2 markers arginase (a, $p < 0.05$) and CD163 (b, $p < 0.05$) is enhanced by posttraumatic hypothermia (4 h). CD206, IL-10, and YM1 also show a trend toward increased expression with hypothermia (c–e) while hypothermia had no effect on the expression of TGF-β (f). RT-PCR: reverse transcription-polymerase chain reaction.

of the innate immune system and represent the first line of defense against injury or disease.[19] After TBI, microglia become activated and demonstrate specific structural and genetic changes that relate to different polarization subtypes including M1-like and M2-like activation phenotypes.[19] In the present study, hypothermia reduced the ratio of iNOS-positive to arginase-positive cells in the isolated CD11b-positive population. This result is consistent with PTH-decreasing iNOS activity and the concept that cooling can also promote reparative processes.[38,39] It should be noted that the M1 phenotype may not be totally bad or the M2 phenotype totally good after brain injury.[40] Future

studies are required to clarify how posttraumatic temperature alters the polarization of microglia/macrophage phenotypes and the establishment of causality in terms of altered traumatic outcome.

In summary, the present study provides new quantitative data emphasizing the complex inflammatory response to TBI and the temperature sensitivity of specific cellular populations. Therapeutic hypothermia introduced early after TBI was shown to produce significant effects on the M1/M2 phenotype balance. This hypothermia-induced polarization of resident microglia and infiltrating blood monocytes to an M2-activated phenotype may participate in the previously
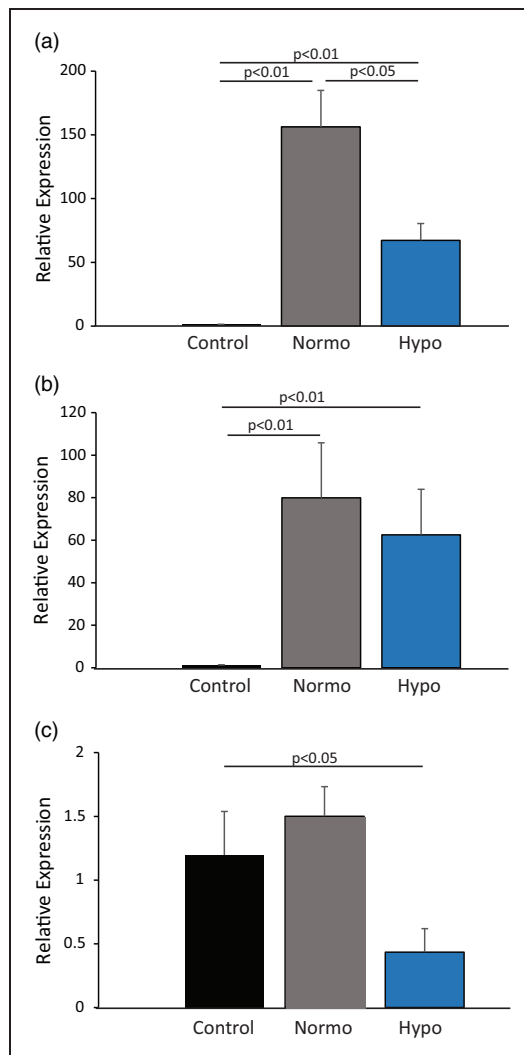
Figure 7. RT-PCR of Ccl2 (MCP1) expression in the ipsilateral cortex (a and b) and hippocampus (c), n = 5 per group. TBI greatly increases the levels of expression of Ccl2 in the cortex at 4 h with a decrease by 24 h by about half p < 0.01. Hypothermia significantly decreases this overexpression at 4 h (a) p < 0.05, but not at 24 h. Hypothermia significantly decreases the level of expression in the hippocampus (c) p < 0.05. (a) 4 h cortex, (b) 24 h cortex, and (c) 4 h hippocampus. MCP1: monocyte chemo-attractant protein 1; RT-PCR: reverse transcription-polymerase chain reaction; TBI: traumatic brain injury.

documented protection of vulnerable cells and the preservation of the microenvironment by restraining endogenous and microvascular inflammatory events. Together, these temperature-sensitive consequences may limit secondary injury processes and promote reparative processes including neurogenesis, oligodendrogenesis, and angiogenesis. Future studies are required to extend these findings to assess optimal therapeutic windows for targeting these complex inflammatory processes with therapeutic interventions including therapeutic hypothermia and targeted temperature management.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

WDD and HMB conceived of and designed the research project, analyzed and interpreted the data, and helped write the manuscript. JST conducted the analysis of the flow cytometry and RT-PCR data and helped write the "Methods" and "Results" sections. JST and HMB also worked on the figures and performed statistical analyses. All authors critically reviewed and approved of the manuscript.

Supplementary material

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data

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